

Specificity of anti-Sm antibodies by ELISA for systemic lupus erythematosus: increased sensitivity of detection using purified peptide antigens

M FIELD, D G WILLIAMS, P CHARLES, AND R N MAINI

From the Clinical Immunology Division, Kennedy Institute of Rheumatology, Hammersmith, London

SUMMARY Sm antigen was purified by immunoaffinity chromatography using a murine monoclonal anti-Sm antibody and was confirmed to be free from contaminating polypeptides. This was then used to detect anti-Sm antibodies in patients' sera by enzyme linked immunosorbent assay (ELISA). Antibodies against Sm were detected in only 9/52 (17%) patients with systemic lupus erythematosus (SLE) by immunodiffusion, but 15/52 (29%) were positive for IgG anti-Sm antibodies by ELISA. The presence of anti-Sm antibodies remained disease specific despite the increase in sensitivity of this assay and validates its potential use for clinical application. There was no correlation between the presence of anti-Sm antibodies and any clinical features of SLE. In 23 renal biopsies a membranous component to the glomerulonephritis correlated with anti-Sm antibodies ($p < 0.05$). Patients from West Africa, the Caribbean Islands, and Asia had a higher prevalence of anti-Sm antibodies than the local Caucasian population.

The overproduction of autoantibodies against nuclear antigens is a cardinal feature of systemic lupus erythematosus (SLE). Those against native double stranded DNA are regarded as pathognomic of this disorder. When originally described, antibodies against the nuclear antigen Sm were also considered diagnostic of SLE,¹ but more sensitive techniques suggested that they may be more widely distributed in autoimmune disease.²

These initial discrepant results arose partly because of poor understanding of antigen structure. Mattioli and Reichlin showed that the Sm antigen could exist as a free moiety or in a complex with another ribonucleoprotein antigen, RNP.³ Hence antibodies to either component of the RNP/Sm antigen could have produced positive results in early assays.

Later studies showed that the Sm antigen could be separated from the RNP/Sm complex by affinity chromatography.⁴ The advent of murine monoclonal anti-Sm antibodies led to the adaptation of this technique to enable characterisation of the RNA and protein components of the Sm antigen.⁵ Immunoblotting analysis with these antibodies⁶ has

confirmed that the antigenic determinants reside on the protein moieties.

The aim of this study was to use a murine monoclonal anti-Sm antibody to obtain highly purified Sm antigen by affinity chromatography for use in an ELISA. Serum samples from patients with autoimmune diseases were analysed for the presence of anti-Sm antibodies to investigate their diagnostic specificity for SLE. The clinical features of all patients were analysed to assess whether the presence of anti-Sm antibodies predisposes to any specific disease manifestations.

Patients and methods

Sm was purified by a modified method of Buchanan *et al.*⁴ Affinity chromatography columns were made by coupling purified IgG to cyanogen bromide activated Sepharose beads (Pharmacia, Milton Keynes, Buckinghamshire). Rabbit thymus extract (Pelfreeze Biologicals, Arkansas, USA) was extracted (2% weight/volume) by sonication for one minute (Soniprep; MSE Scientific Instruments, Crawley, Sussex) in 0.1 M glycine containing 0.35 M NaCl, 10 mM trometamol (TRIS), and 0.1 mM phenylmethanesulphonyl fluoride (pH 7.4). The insoluble fraction was separated by centrifugation at 4000 g.

Accepted for publication 9 March 1988.

Correspondence to Dr M Field, Clinical Immunology Division, Kennedy Institute of Rheumatology, 6 Bute Gardens, Hammersmith, London W6 7DW.

The soluble fraction was initially applied to a column made with normal human IgG, and the drop through was sequentially passed down a column containing IgG from a patient with high titre monospecific anti-RNP antibody and then down the column made with the murine monoclonal anti-Sm antibody (KSm2). Each column was separated, washed, and eluted with 3 M guanidinium hydrochloride (Sigma, Poole, Dorset). Fractions with the highest optical density at 280 nm were pooled, dialysed against phosphate buffered saline (PBS), and stored at 0°C. Each extraction yielded 1–1.5 mg

of pure Sm antigen from 2 g of rabbit thymus extract.

POLYPEPTIDE ANALYSIS

The purified protein antigens were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)⁷ modified according to Williams *et al.*⁶ The separated proteins were stained with Coomassie blue (Fig. 1).

The Sm antigen was also screened by ELISA (which see) using two murine monoclonal antibodies⁶ directed against the 29, 28 kilodalton doublet (KSm5) or the 16 kilodalton (KSm2) components of the Sm antigen to ensure both proteins were present in the purified antigen.

ANTI-Sm ANTIBODY DETECTION

Purified Sm antigen (30 µg/ml) was coated onto ELISA plates (Nunc Immunoplate, Roskilde, Denmark), incubated at 4°C overnight, washed with PBS, and incubated with 2% casein (Fisons, Loughborough, Leicestershire) for 60 minutes at 37°C. The plate was then washed in 0.1% Tween in PBS (PBS/Tween) six times. All subsequent incubations and washes were performed similarly.

Each sample was assayed for anti-Sm antibodies by diluting 1:300 in 0.5% casein in PBS/Tween and applying 50 µl aliquots to individual wells. The plate was then incubated, washed, and bound IgG antibody detected using an antihuman (or antimouse when screening the monoclonal antibodies) IgG conjugate linked to alkaline phosphatase (Sigma, Poole, Dorset). The enzyme was detected with *p*-nitrophenyl phosphate (Substrate 104; Sigma, Poole, Dorset) and the optical density of each sample read at 405 nm in a Titertek multiscan spectrophotometer (Flow Laboratories, Rickmansworth, Hertfordshire).

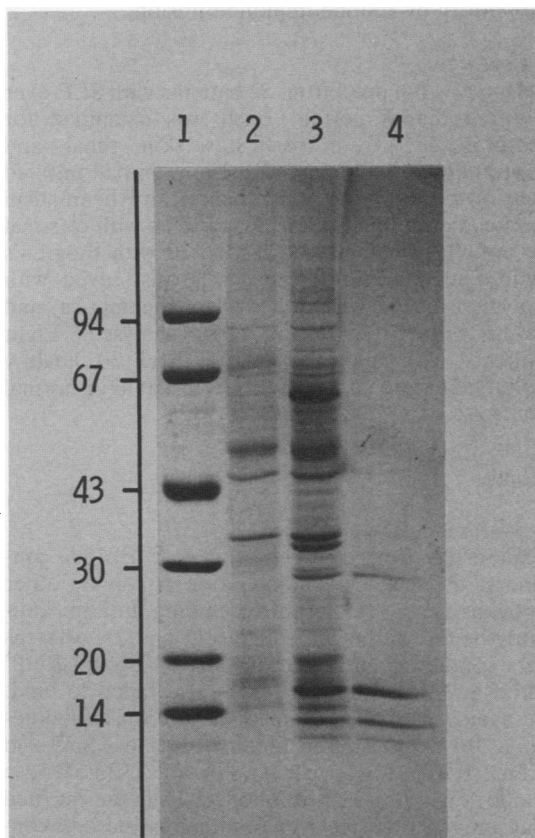


Fig. 1 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of the eluates from the immunoaffinity columns; comparison with the molecular weight standards (mol. wt 94, 67, 43, 30, 20·1, 14·4 kilodaltons) in lane 1. Lane 2 shows the eluate from the normal IgG column. Lane 3 shows the eluate from the anti-RNP column with multiple bands including RNP bands at 67 and 33 and Sm bands at 29, 28, 16, and three bands at 13–11 kilodaltons. Lane 4 shows the purified Sm antigen from the monoclonal antibody and shows only the Sm specific bands.

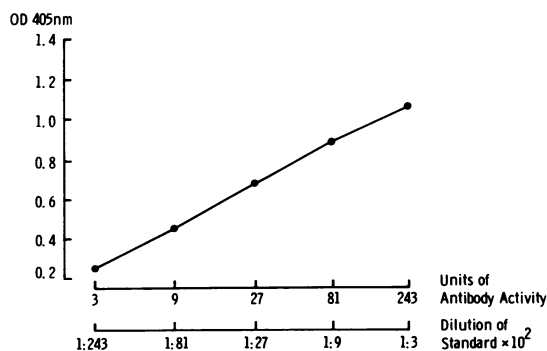


Fig. 2 The construction of the standard curve for the measurement of anti-Sm antibodies (for details see text).

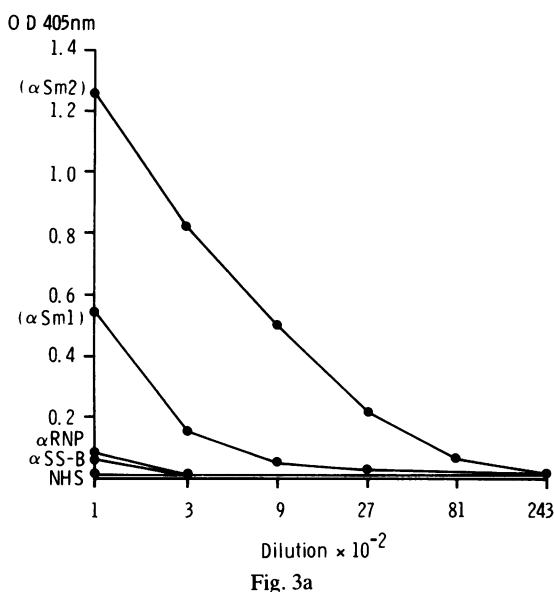


Fig. 3a

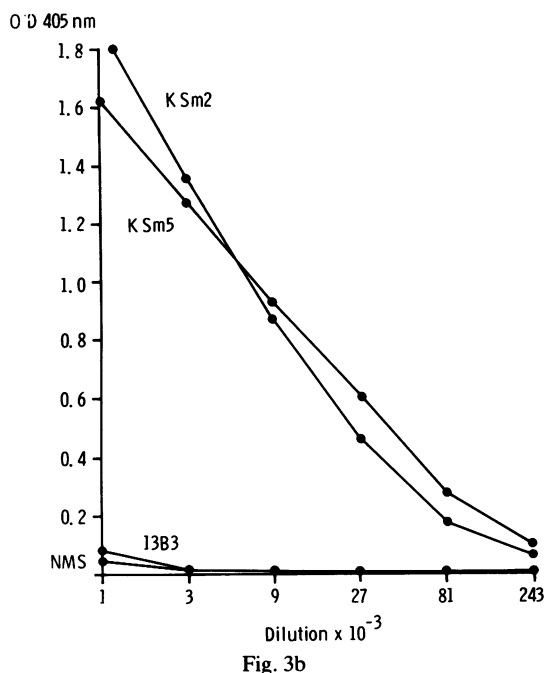


Fig. 3b

Fig. 3 Results from representative ELISA experiments using (a) human sera of defined specificity, demonstrating positive results with serum containing anti-Sm antibody, whereas those with anti-RNP, anti-SS-B activity, and normal human serum do not bind, and (b) murine monoclonal antibodies (KSm5 reacting with the 29, 28 kilodalton doublet and KSm2 binding to the 16 kilodalton Sm antigen) showing binding, whereas those with anti-SS-B activity (13B3/SW5) and normal mouse serum (NMS) do not bind.

The optical density of each sample was compared with a standard curve obtained by diluting a mono-specific serum containing a high titre of anti-Sm antibodies. This was diluted threefold from 1:100 to 1:24 300 on each plate (Fig. 2). The lowest optical density obtained for a dilution of 1:24 300 was arbitrarily allocated a value of 3 ELISA units, that for the dilution of 1:8100—9 units, increasing up the dilution scale so that the value for the 1:300 dilution was ascribed 243 units. For those serum samples producing results above the standard curve a dilution was made and appropriate correction calculated to obtain the result for the standard dilution. All results were compared with those obtained in the laboratory by routine immunodiffusion.

PATIENTS

Serum was obtained from 52 patients with SLE over a three month period. Each was examined for features of SLE, in particular, skin, renal, and central nervous system dysfunction, and all fulfilled four or more criteria of the American Rheumatism Association.⁸ In addition, 53 patients with classical or definite rheumatoid arthritis, 20 with Sjögren's syndrome, 30 with systemic sclerosis, seven with polymyositis, 12 with polymyalgia rheumatica, and seven with osteoarthritis were analysed. Eight patients with multiple sclerosis and 30 healthy volunteers were also screened as diseased or normal controls.

Results

ANTIGEN ANALYSIS

The results from one representative ELISA confirmed the lack of contamination of Sm by other antigens (Fig. 3a). Serum containing anti-Sm antibody bound to the Sm antigens (1 and 2), whereas sera containing antibodies against SS-B³ and RNP⁵ did not. Normal human serum also failed to bind. Screening with the monoclonal anti-Sm antibodies (Fig. 3b) demonstrated binding of both KSm5 and KSm2.⁶ There was no binding of 13B3/SW5, a monoclonal anti-SS-B antibody.⁹ Thus the purified Sm antigen was free from contamination and contained both the 29, 28 kilodalton doublet and the 16 kilodalton epitopes.

SDS-PAGE analysis of the eluate from the antibody columns confirmed the sequential purification of the Sm antigens. The normal IgG column eluate contained some bands purified non-specifically (Fig. 1, lane 2), but the anti-RNP column purified both the 67 and 33 kilodalton bands of RNP together with the 29, 28, 16, 13, and 11 kilodalton Sm polypeptides but also contained many contaminating bands (Fig. 1, lane 3). The anti-Sm

column eluate, however, contained solely the Sm proteins (Fig. 1, lane 4) and had no significant contaminating polypeptides.

DETECTION OF ANTI-Sm ANTIBODIES

In a single time point assay anti-Sm antibodies were detected in 15 patients with SLE (29%) and not in sera from patients with other connective tissue diseases or normal controls (Fig. 4). Comparison of the results obtained by ELISA and immunodiffusion demonstrated broad agreement between the two assays as the patients with higher titre by immunodiffusion tended to have a high titre by ELISA (Table 1). Six patients with SLE, however, had anti-Sm antibodies detectable solely by ELISA.

After a retrospective analysis of all 52 patients a further three had anti-Sm antibodies making a total of 18 (35%). Of the 45 women, 15 were positive (33%), as were three of the seven male patients (43%). Analysis by racial subgroups demonstrated seven patients positive out of 34 Caucasians (21%),

Table 1 Anti-Sm antibodies detected by ELISA and immunodiffusion

Patient No	ELISA (units)*	Immunodiffusion (dilution)†
1	52 650	>1:64
2	1 647	Neg
3	1 273	1:4
4	630	1:1
5	531	Neg
6	282	1:4
7	78	1:4
8	63	1:1
9	62	1:4
10	58	1:4
11	46	Neg
12	20	1:1
13	15	Neg
14	10	Neg
15	6	Neg

*Normal value <3.

†Immunodiffusion was measured as a dilution of serum in which a precipitin could still be seen.

Table 2 Renal biopsy results in patients with anti-Sm antibodies*

Grade	Anti-Sm positive	Anti-Sm negative	p Value
Normal (I)	0	0	NS
Mesangial (IIa, IIb)	3	3	NS
Focal and segmental (III)	2	5	NS
Diffuse proliferative (IV)	0	5	<0.05
Membranous (V)	5†	1	<0.05

*Renal biopsy data were obtained from 23 of the patients with SLE and classified according to Appel *et al.*¹⁰

†Those with a membranous component included two with some evidence of proliferative change and one with a focal change.

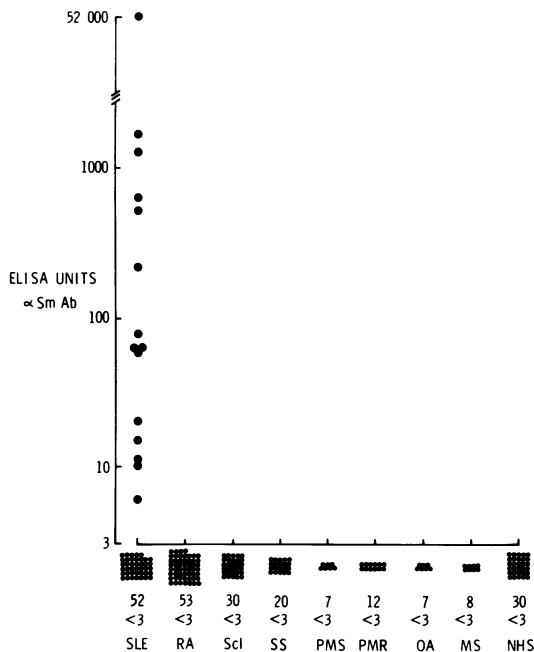


Fig. 4 The prevalence of anti-Sm antibodies in a variety of autoimmune and connective tissue diseases demonstrating their restriction to SLE. SLE=systemic lupus erythematosus; RA=rheumatoid arthritis; Scl=sclerosis; SS=Sjögren's syndrome; PMS=polymyositis; PMR=polymyalgia rheumatica; OA=osteoarthritis; MS=multiple sclerosis; NHS=normal human serum.

eight of 11 Afro-Caribbean patients (73%), and three of seven Asian patients (43%).

The presence of anti-Sm antibodies did not correlate with any features of SLE; in particular there was no correlation with skin, central nervous system, or renal disease. Of the 23 patients in whom renal biopsy was graded,¹⁰ however, the presence of anti-Sm antibodies was negatively associated with a pure diffuse proliferative glomerulonephritis ($p<0.05$) and positively associated with a membranous component to the glomerulonephritis ($p<0.05$) (Table 2).

Discussion

These data demonstrate that immunoaffinity chromatography with monoclonal antibodies produces large quantities of Sm antigen without significant

contamination. The peptide composition of purified Sm compares well with that extracted by affinity chromatography,^{5 11} biochemical purification,¹² and immunoprecipitation.¹³

In an ELISA study for anti-Sm antibodies Maddison *et al* immunopurified Sm from RNase digested thymus extract using human polyclonal antisera.¹⁴ Anti-Sm antibodies were detected in patients with SLE but also in those with rheumatoid arthritis and other autoimmune diseases. Our data show that extraction by human polyclonal antisera leads to copurification of non-specifically bound proteins (Fig. 1), which could result in false positive tests. These data demonstrate that the disease specificity of anti-Sm antibodies for SLE in sensitive ELISA assays depends on antigenic purity.

Pollard and Tan used biochemically purified antigen in an ELISA and found anti-Sm antibodies restricted to patients with SLE, but also in one patient with diabetes mellitus and a facial rash.¹⁵ The use of the monoclonal antibody to purify Sm antigen by affinity chromatography produces larger quantities of pure Sm than biochemical techniques, and its use in the ELISA shows that only patients with SLE have detectable IgG anti-Sm antibodies. Thus this method will provide an assay which could be readily used in clinical practice.

Comparison of ELISA and immunodiffusion showed six samples positive for anti-Sm by ELISA which were negative by immunodiffusion. Of these, four had anti-Sm antibodies in their serum detected by immunodiffusion either in previous or subsequent samples, implying that the ELISA is more sensitive than immunodiffusion. All four samples also had detectable anti-RNP antibodies by immunodiffusion, which may obscure anti-Sm precipitins.¹⁶

Early studies suggested a link between anti-Sm antibodies and cerebral SLE,¹⁷ though these antibodies were not confirmed as markers for central nervous system disease in the lupus population as a whole.¹⁸ Comparison of the 52 patients in this study failed to delineate any association between anti-Sm antibodies and clinical characteristics of SLE. Similarly, no lupus features were ascribed to the presence of anti-Sm antibodies when using immunoprecipitation to detect these antibodies.¹⁹ These increasingly sensitive assays may well include a more representative sample of the lupus population in the positive group, hence removing any dissimilarity between populations.

A pure diffuse proliferative glomerulonephritis was not detected in any patient with anti-Sm antibodies, confirming results from two studies that compared the presence of renal disease with auto-antibody profile. Winn *et al* found only one of 23

patients with anti-Sm antibodies with this severe proliferative renal change,²⁰ and Venables *et al* showed that the presence of antibodies to the soluble cellular antigens in general was commonly noted in association with a membranous glomerulonephritis.²¹ In murine models of SLE only the MRL strain develops anti-Sm antibodies, and these animals have a less severe glomerulonephritis than the NZB/W mice, which only have anti-DNA antibodies.²²

These studies demonstrate a higher incidence of anti-Sm antibodies than the 4–7% reported in the English studies^{23 24} and the 17% in the Indian population²⁵ and are more in line with the prevalence in the USA lupus population.²⁶ Nevertheless, the wide racial difference in the incidence of anti-Sm antibodies has been confirmed.²³ Whether this is the result of genetic differences or the effect of some environmental factor in the initiation of the anti-Sm antibody response remains to be understood.

This work was supported by the Arthritis and Rheumatism Council.

References

- 1 Tan E M, Kunkel H G. Characteristics of a soluble nuclear antigen precipitating with sera of patients with systemic lupus erythematosus. *J Immunol* 1966; **96**: 464–71.
- 2 Sharp G S, Irvin W S, LaRoque R L, *et al*. Association of autoantibodies to different nuclear antigens with clinical patterns of rheumatic diseases and responsiveness to therapy. *J Clin Invest* 1971; **50**: 350–9.
- 3 Mattioli M, Reichlin M. Characterisation of a soluble nuclear ribonucleoprotein antigen reactive with systemic lupus erythematosus sera. *J Immunol* 1971; **107**: 1281–90.
- 4 Buchanan R R C, Venables P J W, Morgan A, Stains N A, Smith P R, Maini R N. Separation of two molecular species of the Sm antigen by affinity chromatography with murine monoclonal and human antinuclear antibodies. *Clin Exp Immunol* 1983; **51**: 8–16.
- 5 Billings P B, Hoch S O. Isolation of intact Sm/RNP antigens from rabbit thymus. *J Immunol* 1983; **131**: 347–51.
- 6 Williams D G, Stocks M R, Smith P R, Venables P J W, Maini R N. Murine lupus monoclonal antibodies define five epitopes on two different Sm polypeptides. *Immunology* 1986; **58**: 495–500.
- 7 Laemmli U K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **227**: 680–5.
- 8 Tan E M, Cohen A S, Freis J F, *et al*. The 1982 revised ARA criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1982; **25**: 1271–7.
- 9 Smith P R, Williams D G, Venables P J W, Maini R N. Monoclonal antibodies to the Sjögren's syndrome associated antigen SS-B (La). *J Immunol Methods* 1985; **77**: 63–76.
- 10 Appel G B, Silva F G, Pirani C L, Meltzer J I, Estes D. Renal involvement in systemic lupus erythematosus. A study of 56 patients emphasising histological classification. *Medicine (Baltimore)* 1978; **57**: 371–410.
- 11 Billings P B, Hoch S O. Characterization of U small nuclear RNA-associated proteins. *J Biol Chem* 1984; **259**: 12850–6.
- 12 Lerner M R, Steitz J A. Antibodies to small nuclear RNAs complexed with proteins are produced by patients with systemic lupus erythematosus. *Proc Natl Acad Sci USA* 1979; **76**: 5495–9.

- 13 Connor G E, Nelsom D, Wisniewolski R, Lahita R G, Blobel G, Kunkel H G. Protein antigens of the RNA-protein complexes detected by anti-Sm and anti-RNP antibodies found in the serum of patients with systemic lupus erythematosus and related disorders. *J Exp Med* 1982; **156**: 1475-85.
- 14 Maddison P J, Skinner R P, Vlachoyiannopoulos P, Brennan D M, Hough D. Antibodies to RNP, Sm, Ro(SSA), La(SSB) detected by ELISA: their specificity and interrelations in connective tissue disease sera. *Clin Exp Immunol* 1985; **62**: 337-45.
- 15 Pollard K M, Tan E M. Purification of the Sm nuclear autoantigen. Detection and clinical significance of IgM antibody. *Clin Exp Immunol* 1985; **60**: 586-96.
- 16 Nakamura R M, Peebles C L, Rubin R L, Molden D P, Tan E M. *Autoantibodies to nuclear antigens (ANA)*. Chicago: American Society of Clinical Pathologists Press, 1985.
- 17 Winfield J B, Brunner C M, Koffler D. Serologic studies in patients with systemic lupus erythematosus and central nervous system dysfunction. *Arthritis Rheum* 1978; **21**: 289-94.
- 18 Barada F A, Andrews B S, Davis J S, Taylor R P. Antibodies to Sm in patients with systemic lupus erythematosus. Correlation of Sm antibody titres with disease activity and other laboratory parameters. *Arthritis Rheum* 1981; **24**: 1236-44.
- 19 Williamson G G, Pennebaker J, Boyle J A. Clinical characteristics of patients with rheumatic disorders who possess antibodies against ribonucleoprotein particles. *Arthritis Rheum* 1983; **26**: 509-15.
- 20 Winn D M, Wolfe J F, Lindberg D A, Fristoe F H, Kinsland L, Sharp G C. Identification of a clinical subset of systemic lupus erythematosus by antibodies to the Sm antigen. *Arthritis Rheum* 1979; **22**: 1334-7.
- 21 Venables P J W, Tung Y, Woodrow D F, Moss J, Maini R N. Relationship of precipitating antibodies to soluble cellular antigens and histologically defined renal lesions in systemic lupus erythematosus. *Ann Rheum Dis* 1983; **42**: 17-22.
- 22 Andrews B S, Eisenberg R A, Theofilopoulos A N, *et al*. Spontaneous murine lupus like syndromes. Clinical and immunopathological manifestations in several strains. *J Exp Med* 1978; **148**: 1198-215.
- 23 Bernstein R M, Bunn C C, Hughes G R V, Francoeur A M, Mathews M B. Cellular protein and RNA antigens in autoimmune disease. *Mol Biol Med* 1984; **2**: 105-20.
- 24 Gordon T, Maddison P J, Isenberg D A. Rapid development of cerebral atrophy in systemic lupus erythematosus. *Br J Rheumatol* 1986; **25**: 296-9.
- 25 Malaviya A N, Misra R, Bannerjee S, *et al*. Systemic lupus erythematosus in North Indian Asians. A prospective analysis of clinical and immunological features. *Rheumatol Int* 1986; **6**: 97-102.
- 26 Notman D D, Kurata N, Tan E M. Profiles of antinuclear antibodies in systemic rheumatic diseases. *Ann Intern Med* 1975; **83**: 464-9.